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Maximum microvessel density (MMVD) in primary tumors is a sensitive prognostic indicator for many solid tumors, including breast. These data imply functional significance for the "hot spots" of microvessels which are quantitated in MMVD determinations. Since prognosis is determined by metastasis, functional significance of the "hot spots" in the process of metastasis is also implied. We hypothesize that the neovascular "hot spots" are the sites of metastasis and that gene expression in the tumor in the vicinity of the "hot spot" is important in the generation of the "hot spot" and in the process of metastasis. In addition to our previous result that metastasis is correlated to tumor size, we have found a relation between metastasis and MMVD. We have developed a method to make sense amplified RNA from 1 pg of RNA. Therefore we have combined amplified RNA production with the Clontech method of template switching and long-distance polymerase chain reaction (PCR). We are able to produce quantities of copy DNA which can be used for analysis of differential gene expression.

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Sandra W. McLeroy 4/13/99

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INTRODUCTION

This proposal combines microvessel density measurements with microdissection in an effort to identify genes differentially expressed by tumor cells in "hot spots" of maximal microvessel density. In order to find the "hottest spot' in each tumor, we screened representative sections from all regions of each tumor. We quantified these spots to see whether a particular area of the tumor has "hotter" spots of microvessels and related these data to the degree of lung metastasis found in the host. Finding the "hottest spot" in each tumor will enable us to microdissect those areas in highly metastatic tumors and compare them to areas not associated with high microvessel density with regard to differential gene expression.

Before we are able to perform expression analysis on our tumor specimens, we first have to be able to purify and amplify RNA from the dissected cells, and make cDNA. Since extremely small amounts of source RNA will be harvested, the cDNA will have to be amplified. As mentioned in the previous report, there are two concerns for any method of amplification: fidelity and yield. Since the last report, we have been working to improve our methods of amplification. This report contains a scheme, which combines the Clontech method of cDNA synthesis and amplification [template switching (1) followed by long-distance PCR(2)] with a new method of amplified RNA, developed in our laboratory.

For differential analysis of gene expression we are currently using the amplified length polymorphism analysis (AFLP) (3) on cDNA in connection with another project in our laboratory. Because of our great experience with this method, we had planned to use it with this project also. However, the recent availability of microarray technology (4) has made differential analysis of gene expression for known genes extremely fast. Therefore we are also considering the use of microarray technology in collaboration with Dr. Yan Su, at the Georgetown University.

BODY

Aim 1. We will identify critical microenvironments in the tumors produced by FGF-1 transfected MCF-7 cells in nude mice by sensitively and accurately correlating the degree of metastasis in the lungs and lymph nodes with maximal microvessel density in the hottest spot in each tumor.

In last year's report we were in the process of scoring the representative PECAM-1 stained sections from all tumors. We had scored most of the 30 tumors for the A1 slice and many of the tumors from other slices. Our goal in this analysis is to identify the "hottest spot" in the most metastatic tumors so as to use those areas for microdissection. Important issues of quantitating the microvessel density (MVD) are: 1.selection of the tissue sample as part of the whole tumor specimen; 2.tissue processing and immunostaining; 3.selection of the area for microvessel enumeration and 4.the technique of MVD evaluation. As described in our previous report, tumors were cut in a standardized way into four transverse and four longitudinal slices, 3-4 mm thick, frozen and sectioned serially onto uncoated slides. Slides were stained for PECAM-1, a specific staining for endothelial cells, described in our previous report.

We used the method of Weidner *et al.* 1991 (5), modified according to Vermeulen *et al.* 1996 (6), which utilizes a Chalkley graticule. Only short, sprouting, vessels without lumens are considered, mostly located in the edge of the tumor. Representative sections from all regions of each tumor were screened under low power (40x) for the area with the highest MVD in the tumor. This MVD was then quantified at 200x magnification. By counting the dots of the graticule coinciding with PECAM positive microvessels, a Chalkley score was determined for each tumor (Table 1). The higher the MVD was for that tumor, the higher the Chalkley score. If more than one candidate area of maximal MVD was identified in the screening step, then the Chalkley score was determined for each and the highest score was used. We were able to find a relation between highest MVD scored in each tumor and the number of lung metastases in the host (Fig.1). Except for one case, we found low MVD related to low number of lung metastases. All tumors with higher numbers of metastases had higher maximal MVD's, but not all tumors with a high MVD had a high number of lung metastases. In the coming year, areas of highest MVD in tumors with highest number of metastasis will be microdissected and

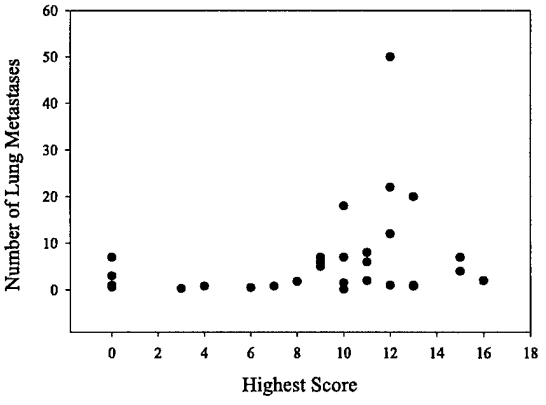

Figure 1. Relationship of lung metastases and Chalkley score. The area of maximal microvessel density for each tumor was quantified with a Chalkley graticule. Lung metastases were quantified by image analysis (see previous report for method).

extracted RNA used to identify genes that may be involved in metastatic processes. These genes would be those differentially expressed in tumor cells adjacent to hot spots when compared to areas of tumor not associated with hot spots.

Aim 2. We will analyze differential gene expression in tumor cells in, the area of hot spots, by microdissection followed by differential display PCR.

Laser microdissection will be used to microdissect tumor cells in the area of the hottest spot. A hot spot area is first selected under the microscope at low magnification (100x) and the cells (a spot of ≈ 30 μm) are "burned" onto a special plastic cap adjusted right on top of this area, by activating the laser beam. As mentioned in the previous report, we have practiced this technique and are able to obtain RNA from it.

For making cDNA from RNA obtained by laser microdissection, we attempted the Clontech method, described in the previous report, utilizing the SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA). This method combines template switching with long-distance PCR (7). This approach was not successful, possibly due to the small amount of RNA isolated. Therefore we modified the aRNA method described in our previous report, to produce sense rather than antisense aRNA (below).

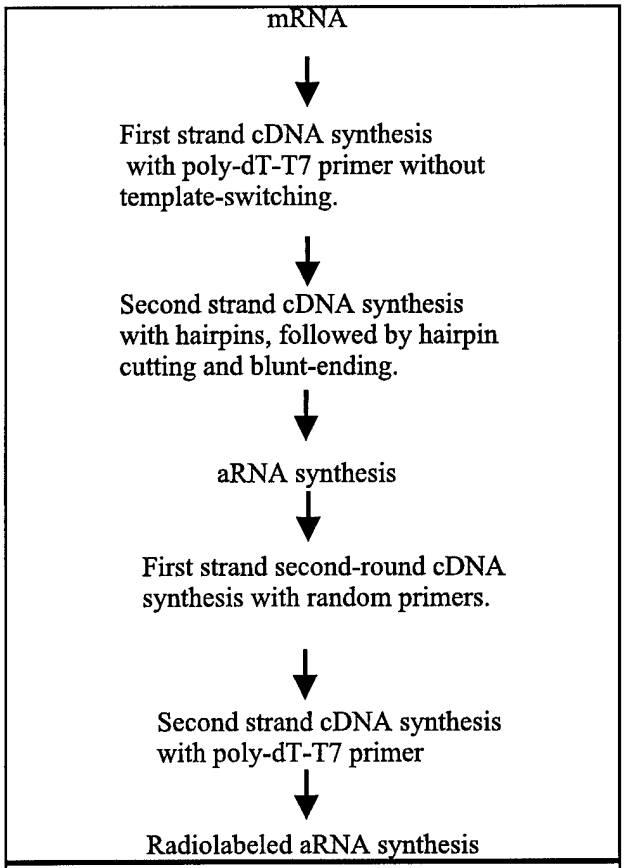

Figure 2. Scheme for "conventional" aRNA synthesis.

The "conventional" amplified RNA (aRNA) method as described in the previous report, appends a T7 RNA polymerase promoter sequence to the poly-dT primer used for first strand cDNA synthesis. Problems with the "conventional" aRNA approach as presently implemented include the antisense orientation of the aRNA and the less then full-length species it generates. This product is ideal for use as a probe since it can be easily labeled with radionucleotides at the time of synthesis, but it cannot be used for RT-PCR unless reversed primers (antisense instead of sense and sense instead of antisense) are synthesized. Moreover, although the conditions used to generate the aRNA

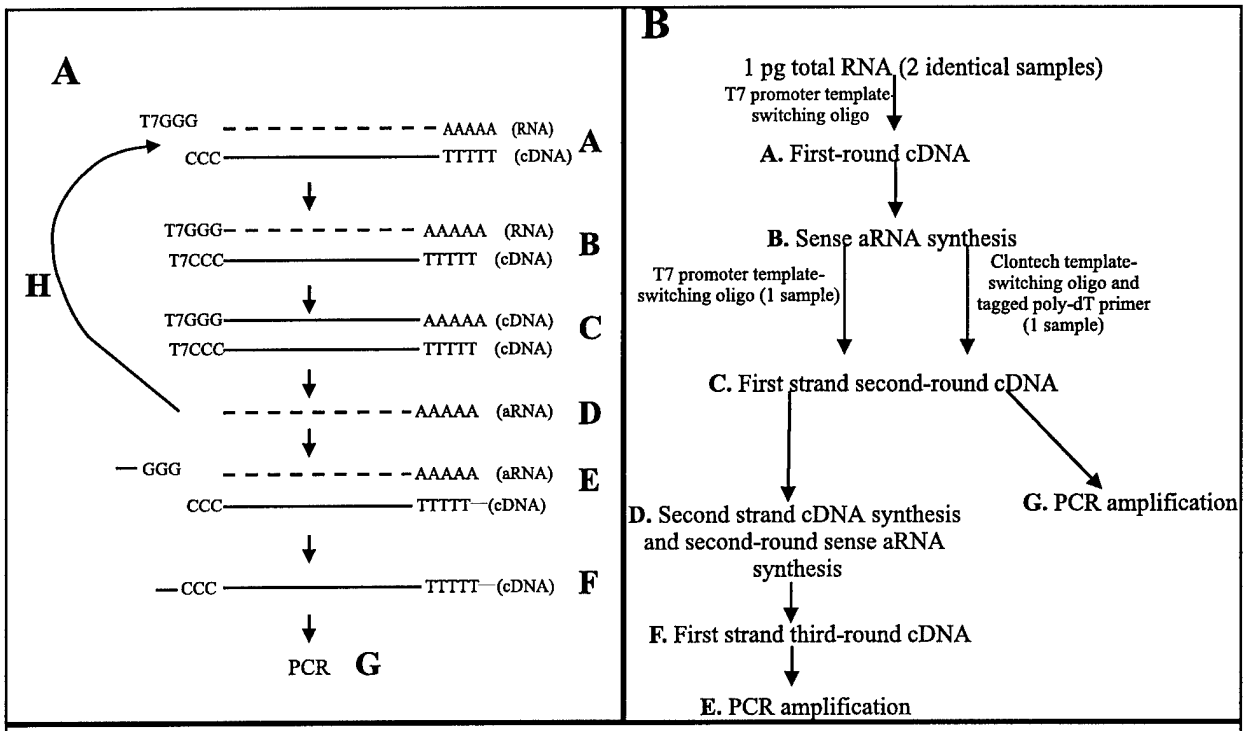

Figure 3. Scheme for combination of template switching, aRNA production and SMART™ cDNA synthesis. A. Scheme for appending the T7 promoter sequences to the 3'end of the first strand cDNA and generation of sense aRNA. **B.** Flow diagram for an experiment comparing yield of SMART™ cDNA after one or two rounds of sense aRNA production.

favor a high degree of synthesis, information concerning the length of individual species has not been obtained. aRNA can be used to generate second-round cDNA, but because of the antisense orientation, a poly-dT primer cannot be used to prime the reverse transcription reaction. Therefore, random primers are used to prime first strand cDNA synthesis, followed by poly-dT priming of second strand synthesis [Figure 2 and (8,9)]. This guarantees that the second-round cDNA will not be full length.

Using the method of template switching, we were able to make sense aRNA from 1 pg of total RNA. This was accomplished by using a poly-dT primer with a template- switching oligonucleotide incorporating the T7 RNA polymerase promoter for 1st strand cDNA synthesis as described below. We have combined sense aRNA production with the Clontech method of template switching and long-distance PCR to produce quantities of cDNA which can be used for analysis of differential gene expression.

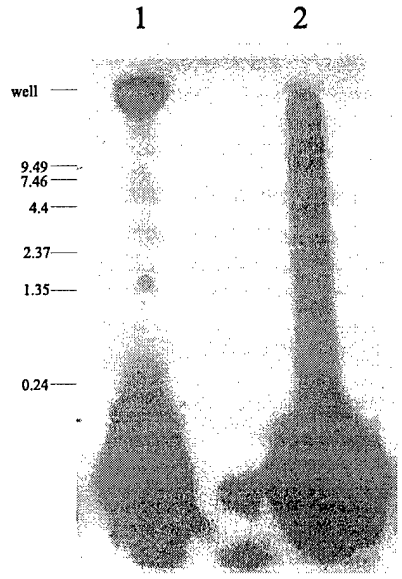

Figure 4. Second-round sense aRNA production. An identical cDNA sample to that produced in Figure 3B, step D was used to produce aRNA incorporating ^{32}P-labeled UTP. The labeled RNA is shown in lane 2 of the autoradiogram. Lane 1 is the product of a similar aRNA reaction which lacked T7 RNA polymerase.

Production of sense aRNA. Total RNA (1 pg) was used as template for a reverse transcription reaction with a poly-dT primer and Superscript II (Life Technologies, Gaithersburg MD) as the RT (step A and B in Figure 2A). The single-stranded template-switching oligonucleotide included in the reaction had four 3' dGs with T7 RNA polymerase promoter sequences appended 5' to the dGs. This same oligonucleotide was used to prime the second strand reaction, yielding double stranded cDNA with an intact T7 RNA polymerase promoter sequence at the 5' end (step C of Figure 3A). Amplified RNA was then synthesized with T7 RNA polymerase, yielding full length sense RNA (step D in Figure 3A). Since the transcription start site for T7 RNA polymerase includes only the terminal 6 nucleotides of the promoter, which in this case would be followed by the 4 Gs used for template switching, the sense aRNA generated by this procedure would have 10 additional nucleotides at the 5' end, when compared to the original mRNA.

The first round aRNA generated above was then used for second round cDNA synthesis in 2 ways. First, the first-round aRNA was used with a template-switching, long range PCR protocol to generate PCR-amplified cDNA (Steps E, F and G in Figure 3A). Second, the first-round aRNA was used as a template for reverse transcription followed by second strand synthesis in the same way as the original cDNA was synthesized (Step H in Figure 3A). This produced second-round cDNA, which could be used to produce second-round aRNA. The second-round aRNA was then used as template with the template-switching, long range PCR protocol to generate PCR-amplified cDNA. An overall scheme of the whole experiment is shown in Figure 3B.

Figure 4, lane 2 shows ^{32}P-labeled UTP incorporation into second-round aRNA produced in this manner (the aRNA was produced in step D, Figure 3B). Scintillation counting of trichloroacetic acid precipitable material from a portion of this sample allowed us to estimate that the reaction produced 1.5 ng of aRNA. If we assume that 5% of total RNA is mRNA, from 1 pg of total RNA, we had 50 fg of starting mRNA, producing a 30,000-fold final amplification. This yield is somewhat short of the million-fold amplification predicted for two rounds of "conventional" aRNA synthesis (9). This may be because our scheme appends the T7 promoter at the 5' end of the first round cDNA,

so shorter than full-length cDNAs will not have the T7 promoter sequences and will not be able to serve as template for aRNA transcription. In contrast, the "conventional" aRNA protocol, which appends the T7 promoter sequence to the poly-dT primer, nearly every product of first strand synthesis, even extremely short ones, will produce aRNA. This selection for short species is increased in second-round aRNA synthesis since random priming of second-round first strand synthesis again produces shorter species. Considering that we have set up our conditions such that incomplete first strands will not give rise to cDNAs which will produce aRNA, a 30-fold reduction in yield is a small price to pay in exchange for longer cDNAs.

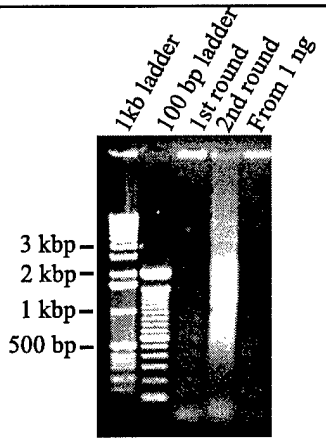

Figure 5. cDNA produced by the SMART™cDNA synthesis method from 1st and 2nd round aRNA. Ethidium bromide staining of a 1% agarose gel of cDNA generated by the SMART™cDNA synthesis method from first round sense aRNA (step B, Figure 3B) in lane 3 or second round sense aRNA (step D, Figure 3B) in lane 4 using 1 pg of total RNA as starting material. Lane 5 contains cDNA produced by the SMART™cDNA synthesis method directly from 1 ng total RNA.

Synthesis of cDNA from sense aRNA. Figure 5 shows an ethidium bromide stained agarose gel of cDNA synthesized with the template-switching, long-range PCR method (26 cycles) from aRNA produced from first-round cDNA synthesis in lane 3 (step G in Figure 3B), and from second-round cDNA synthesis in lane 4 (step E in Figure 3B), each produced from 1 pg total RNA as starting material. For comparison, cDNA synthesized directly from 1 ng total RNA (the minimum recommended template is 50 ng) as starting material is shown in the last lane. It should be noted that approximately equal yields of cDNA are obtained from 1 ng of total RNA and from 1 pg total RNA which has undergone one round of aRNA synthesis. Moreover, the intensity of ethidium bromide staining seems to peak around 1.5 kb and extends into very high molecular weights. Therefore, we are optimistic that this cDNA is of high quality and contains long transcripts.

The gel depicted in Figure 5 contains approximately 30% of the cDNA produced in the synthesis reaction. We therefore feel that this method of cDNA synthesis, when applied to RNA purified from microdissected samples, will enable us to perform analysis of differential gene expression as described in the proposal.

Analysis of differential gene expression. As mentioned in the previous report, we are currently performing amplified fragment-length polymorphism analysis (AFLP) on cDNA obtained from tumor-derived endothelial cells in connection with another project in the laboratory. We currently have eight candidate differentially expressed genes resulting from that analysis. Since we are experienced with that method, we could use it in this project also. However, since this proposal was funded, microarray technology has become more universally available. We also have a

collaborator at Georgetown, Dr. Yan Su, who has worked in the past with Dr. Jeff Trent of NIH, one of the originators of the microarray technique. In addition mouse microarrays are being developed during the next year. As we obtain cDNA from the areas of hot spots, we will be working with Dr. Su to develop a microarray application of this project.

CONCLUSION

Scientific conclusions to date:

1. We are able to find the "hottest spot" in each tumor and quantitate the vessels in a standardized way. In addition to our previous finding that degree of pulmonary micrometastasis is tightly correlated with tumor size, our recent data confirm a relation between degree of pulmonary micrometastasis and highest MVD scored in each breast tumor.

2. We are able to make sense aRNA from 1 pg of RNA, by using the method of template-switching. We have combined sense aRNA production with the Clontech method of template switching and long-distance PCR, to produce quantities of cDNA which can be used for analysis of differential gene expression. We are very optimistic that this cDNA is of high quality and contains long transcripts.

DISCUSSION

As we have mentioned in the previous report, this research is by definition risky, however, we were able to find a relation between microvessel density in the tumor and the degree of lung metastasis in the host. Moreover, we were able to purify RNA and we have modified the methods of RNA amplification and synthesis of cDNA, enabling us to make cDNA from 1 pg of purified RNA. What we have achieved so far brings us closer to our goal to microdissect tumor cells adjacent to hottest spot and compare these with non-hotspot associated tumor cells, by using differential gene expression methods. As the microarray technology is now within our reach, our research, although risky, looks even more promising than before.

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APPENDIX

LIST OF ABBREVIATIONS AND ACRONYMS

aRNA	amplified ribonucleic acid
AFLP	amplified fragment-length polymorphism
cDNA	copy DNA
DNA	deoxyribonucleic acid
MVD	microvessel density
PCR	polymerase chain reaction
PECAM-1	platelet-endothelial cell adhesion molecule 1
RNA	ribonucleic acid
RT-PCR	reverse transcription followed by the polymerase chain reaction
T7	bacteriophage T7

Meeting abstracts since the last report: None for this project.

Publications since the last report: None in connection with this project.

Manuscripts in preparation: None to date.

Personnel receiving pay from this negotiated effort during this period:

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